SCIENCE CHINA
Life Sciences

SPECIAL TOPIC: Model animals and their applications
• RESEARCH PAPER •

April 2015 Vol.58 No.4: 368–372
doi: 10.1007/s11427-015-4828-1

Inactivation of *Cipc* alters the expression of *Per1* but not circadian rhythms in mice

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Received September 30, 2014; accepted January 18, 2015

Circadian clocks are comprised of self-sustained transcriptional/translational feedback loops, which regulate the rhythms of physiology and behavior in mammals. CLOCK-interacting protein, Circadian (CIPC), has been indicated as an additional negative-feedback regulator of the circadian clock *in vitro*, although its physiological roles in circadian clock are unknown. Here, we generated *Cipc* homozygous knockout ($Cipc^{-/-}$) mice and assessed the resultant circadian phenotypes. Surprisingly, the mRNA expression profiles of core clock genes in the liver of $Cipc^{-/-}$ mice showed no significant differences from that in wild-type mice except for *Per1*. $Cipc^{-/-}$ mice displayed normal locomotor rhythm and entrained activity pattern in both 12:12 light-dark cycle and constant dark cycle. Furthermore, deletion of *Cipc* in lungs and adipose tissues did not influence their peripheral clock. The results from this work provided more conclusive data suggesting that CIPC is not critically required for basic clock function.

CIPC, circadian clock, animal model

Citation: Qu ZP, Wang XH, Liu DC, Gao X, Xu Y. Inactivation of *Cipc* alters the expression of *Per1* but not circadian rhythms in mice. Sci China Life Sci, 2015, 58: 368–372, doi: 10.1007/s11427-015-4828-1

Circadian clocks generate circadian rhythms with a period about 24 h, and regulate the physiology and behavior of organisms to adapt to the light/dark cycle caused by rotation of the earth [1,2]. In mammals, at the molecular level, the current model features that circadian clocks are composed of an auto-regulatory transcriptional/translational feedback loops [3]. Specifically, two basic helix-loop-helix transcription factors, CLOCK and BMAL1 form heterodimers and activate the transcription of *Per* (*Per1-3*) and *Cry* (*Cry1-2*) genes. Then PER and CRY proteins inhibit their own transcription by interacting with BMAL1-CLOCK heterodimer complexes [4,5]. Moreover, nuclear receptors REV-ERBα/β and RORα strictly control the expression of *Bmal1* [6–8].

Previous studies identified a new regulator of circadian clock, CIPC, which interacts with CLOCK and represses the CLOCK-BMAL1-mediated transactivation by yeast two-hybrid screening [9]. Both knockdown of endogenous *Cipc* and overexpression of full length *Cipc* resulted in shorter circadian period lengths in fibroblast cells [9].

Here, we used the *Cre-LoxP* system to disrupt the *Cipc* gene and estimated the effects of lacking CIPC on circadian clock. Unexpectedly, although the expression of *Per1* mRNA was decreased, $Cipc^{-/-}$ mice retained normal circadian behavioral rhythms and displayed comparable extents of phase shift upon light stimulus. In addition, the periods of PER2:LUC expression were not significantly altered in lung and adipose tissues from $Cipc^{-/-}$ mice. Thus, our studies demonstrated that CIPC is dispensable for circadian clock.

1 Materials and methods

1.1 Animals and behavioral analysis

Animal studies were performed with an approved protocol

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from the Animal Care and Use Committee of the Model Animal Research Center, Nanjing University. All mice were housed in a 12:12 light-dark cycle under SPF animal facility. For wheel-running activity assay, adult mice were initially entrained on a 12:12 LD cycle for at least 7 d followed by constant darkness for several weeks. The wheel-running period of mice was analyzed by Clocklab as previously described. [10]. For behavior response to light stimulus assay, mice were initially entrained on a 12:12 LD cycle for at least 7 d, followed by DD for one day, and then subjected to a 6-h light pulse at CT (circadian time) 12 or CT18, followed by constant darkness. The activities of mice were recorded by wheel-running.

1.2 Generation of *Cipc* knockout mice

The targeting strategy for *Cipc* was described in Figure 1. By homologous recombination, one LoxP was inserted into intron 2-3, another after 3′UTR. Positive ES cell clones were confirmed by long-range PCR, and injected into C57BL/6J blastocysts to generate chimeric mice. Chimeric mice were crossed with *EIIa-Cre* mice to generate *Cipc* heterozygous ($Cipc^{+/-}$) mice. The heterozygous offspring were continuously backcrossed with C57BL/6J mice for at least six generations and intercrossed to generate $Cipc^{-/-}$ mice.

1.3 RNA isolation, RT-PCR and quantitative PCR

Wild-type and $Cipc^{-/-}$ mice were initially entrained to 12:12 LD cycle for at least 7 d, and then released into constant darkness. Liver was collected at the CT0, 4, 8, 12, 16 and 20 on the first day of DD. Total RNA was extracted from tissues by Trizol reagent (Life Technologies, USA), and reverse-transcribed into complementary DNA (cDNA) with PrimeScript RT-PCR Kit (TaKaRa, Japan). RNA expression was quantified by StepOne Plus real time PCR system (Life Technologies) with SYBR Green reagent (TaKaRa). All the real-time PCR primers were reported as earlier [11].

1.4 Real-time bioluminescence recording

Mice of indicated genotype were maintained in a 12:12 LD cycle for at least 7 d. The explants were prepared within 1 h before dark onset and cultured in D2902 medium (D2902, 0.035% $NaHCO_3$, 10 mmol L^{-1} hepes, 25 U mL^{-1} penicillin, 25 U mL^{-1} streptomycin, 5% fetal bovine serum, 0.1 mmol L^{-1} luciferin) as previously described [12]. Bioluminescence was recorded and analyzed by Lumicycle (Actimetrics, USA).

2 Results

2.1 Generation of $Cipc^{-/-}$ mice

To elucidate the functional roles of CIPC in circadian clock, we first created *Cipc* knockout mice. Due to alternative translational initiation sites, there are five transcripts of mouse *Cipc* from NCBI database. The full-length mouse CIPC contains 411-amino acid rather than 397-aa in previous reports [9] (http://www.ncbi.nlm.nih.gov/gene/217732). In the present targeting strategy, the 3rd and 4th exons including 3′UTR are flanked by loxP sites in the flox allele (Figure 1A). These deleted exons encode 352 aa of CIPC

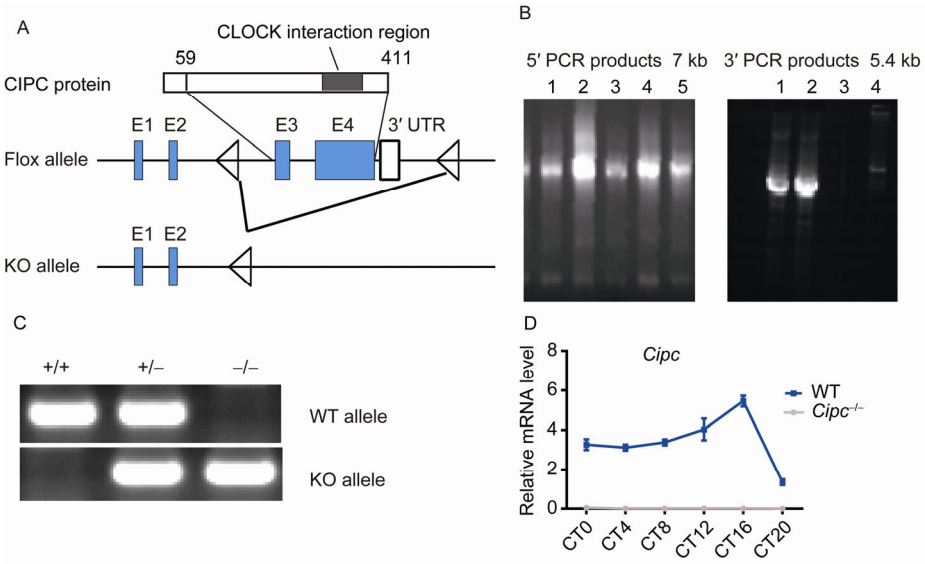

Figure 1 (color online) Generation of $Cipc^{-/-}$ mice. A, Targeting strategies for LoxP sites. One LoxP site was placed in intron 2, another after 3′ UTR. B, Long range PCR screen showing products corresponding to correct recombination in ES cells. C, PCR analysis of tail genomic DNA for wild-type and *Cipc* knockout alleles in wild-type, heterozygous and homozygous knockout mice. D, Quantitative real-time PCR showing the circadian oscillation of *Cipc* mRNA level in the liver, which was completely ablated in $Cipc^{-/-}$ mice.

protein, including CLOCK-interaction region CIPC$^{318–367}$ discovered by yeast two-hybrid assay [9]. The correct targeting ES clones were screened by long range PCR (Figure 1B), and injected into C57BL/6J blastocysts to generate chimeric mice. Chimeric male mice were crossed with *EIIa-Cre* females to generate heterozygous knockout. The heterozygous offspring were continuously backcrossed to C57BL/6J mice for at least six generations, and intercrossed to generate *Cipc* knockout mice confirmed by PCR (Figure 1C).

Robust circadian oscillation of *Cipc* mRNA and protein in the liver has been reported, with a peak at CT14 [9], similar to the present result in wild-type mice (Figure 1D). The deletion of the *Cipc* gene was validated at the RNA level by quantitative real-time PCR (Q-PCR) (Figure 1D). The *Cipc*$^{-/-}$ offspring were born in a Mendelian distribution and all the *Cipc*$^{-/-}$ mice were normal in appearance.

2.2 Loss of *Cipc* has subtle effects on the molecular circadian clock

Firstly, we examined transcriptional levels of core clock genes and clock-controlled gene. Livers were collected from wild-type mice and *Cipc*$^{-/-}$ mice at 4-h intervals in DD. Gene expression levels were quantified by quantitative real-time PCR. In wild-type mice, the transcripts of core clock genes and clock-controlled gene were consistent with previous reports (Figure 2) [11]. Core clock genes, including *Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per2*, *Per3*, *Rev-erba* and clock-controlled gene *Dbp*, displayed comparable expression patterns in *Cipc*$^{-/-}$ and wild-type mice. Only the peak of *Per1* in *Cipc*$^{-/-}$ mice was reduced to half level of wild-type mice (Figure 2).

2.3 *Cipc*$^{-/-}$ mice maintain normal locomotor activity rhythms

To examine the effects of lacking CIPC on circadian behavioral rhythmicity, we recorded the wheel-running activities of *Cipc*$^{-/-}$ mice and their wild-type littermates under constant darkness. *Cipc*$^{-/-}$ mice sustained robust circadian rhythm of activity in LD cycle and constant darkness (Figure 3A). Under constant darkness, the homozygous *Cipc*$^{-/-}$ mice displayed a period of 23.78±0.27 h (mean± standard deviation (SD), n=15), similar to that in wild-type siblings (23.73±0.19 h, n=10) (Figure 3B). Thus, these data suggest that CIPC is dispensable for the generation of robust circadian rhythms, or the proper circadian period in mice.

2.4 *Cipc*$^{-/-}$ mice display normal behavioral responses to light

Mammalian circadian clocks are highly sensitive to light.

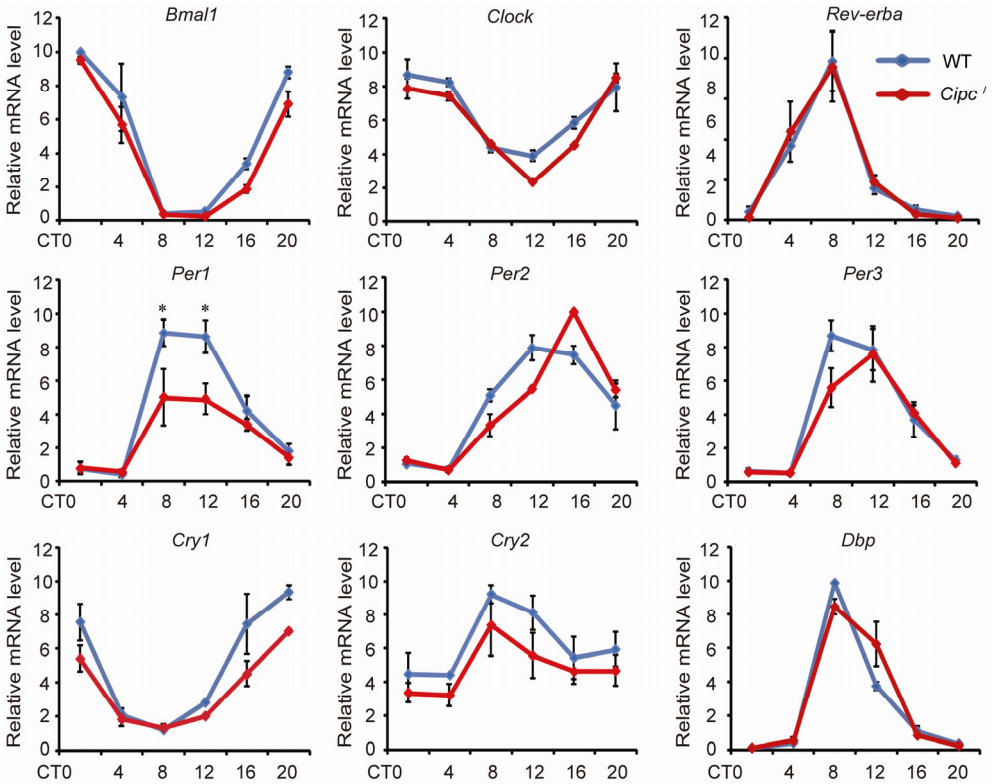

Figure 2 (color online) Influences of CIPC on the molecular circadian clock. Expressions of core clock genes and clock-controlled gene in the liver from wild-type and *Cipc*$^{-/-}$ mice. The relative levels of RNA were analyzed by quantitative real-time PCR and normalized by *Gapdh*.

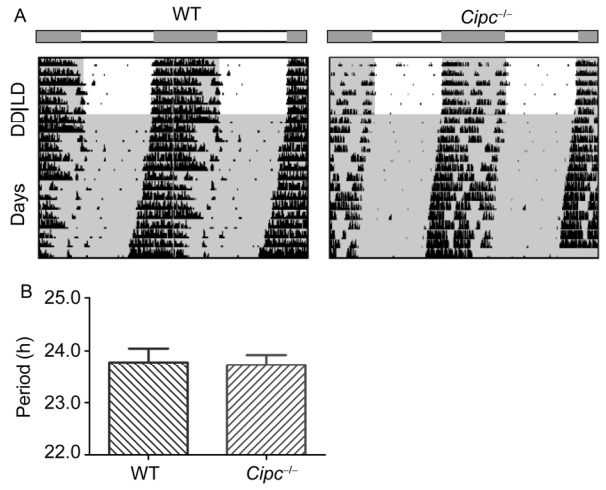

Figure 3 Locomotor activity of wild-type and *Cipc*$^{-/-}$ mice. A, Representative actograms of the wheel-running activity of wild-type and *Cipc*$^{-/-}$ mice. Light bars indicate activity during the light phase and dark bars indicate activity during the dark phase. B, Quantification of the circadian periods from wheel-running activities in constant darkness (23.73±0.19 h (mean±SD, n=10)) and *Cipc*$^{-/-}$ mice (23.78±0.27 h, n=15).

Specifically, an early-night light pulse delays the locomotor activity phase; whereas, a light pulse in the late night advances the locomotor activity phase [1]. To investigate whether the CIPC participates in light response pathways, we analyzed activity phase shifts of *Cipc*$^{-/-}$ mice with a light stimulus in the early night or late night.

Wild-type and *Cipc*$^{-/-}$ mice were entrained in a 12:12 LD cycle for at least 7 d, followed by constant darkness. For the first day of DD, a 6-h light pulse was administrated on either CT12 or CT18. Wheel running activity was recorded and the extent of phase shift was analyzed by the onset of activity. Both wild-type and *Cipc*$^{-/-}$ mice displayed the same extent of phase shift of activity upon the 6-h light stimulus at CT12 or CT18 (6-h light pulse at CT12: WT: 3.18±0.41 h, *Cipc*$^{-/-}$: 3.71±0.46 h; 6-h light pulse at CT18: WT: −1.51±0.53 h, *Cipc*$^{-/-}$: −1.36±0.63 h), respectively (Figure 4). Therefore, these data imply that CIPC is not involved in light response pathways in suprachiasmatic nuclei (SCN).

2.5 Circadian rhythms in lung and adipose explants are not altered in *Cipc*$^{-/-}$ mice

Since loss of CIPC has no notable effect on circadian rhythm of locomotor activity, which is organized by master clock in SCN, we then examined whether CIPC has a role in the peripheral clock.

We monitored the circadian rhythms of peripheral tissues by crossing the *Cipc*$^{+/-}$ mice with the *mPer2*^{Luc} knockin reporter mice [12]. We assessed the luminescence of lung and adipose explants from *Cipc*$^{+/+}$/*mPer2*^{Luc} mice and *Cipc*$^{-/-}$/*mPer2*^{Luc} mice. The periods of PER2:LUC expression in lung and adipose tissues were comparable in wild-type and *Cipc*$^{-/-}$ mice (Lung: WT: 22.23±0.03 h, *Cipc*$^{-/-}$: 22.47±0.27 h; P=0.214. Adipose: WT: 24.48±0.30 h, *Cipc*$^{-/-}$: 24.56±0.36 h; P=0.702) (Figure 5). Together with the wheel-running results, these data suggest that CIPC is not required for the regulation of circadian period.

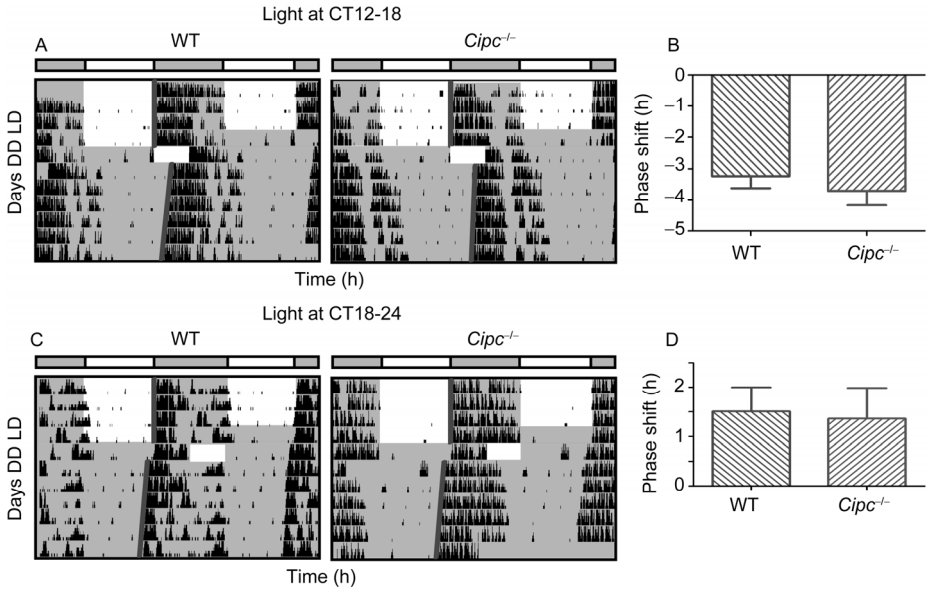

Figure 4 Light responses of wild-type and *Cipc*$^{-/-}$ mice. Mice were initially entrained on a 12:12 LD cycle, followed by constant darkness for 1 d, and then subjected into light pulse during CT12–18 (A and B) or CT18–24 (C and D) followed by constant darkness. The activities of wild-type and *Cipc*$^{-/-}$ mice were recorded by wheel-running system. B and D, Quantification of the phase shifts for wild-type and *Cipc*$^{-/-}$ mice. Negative numbers are phase delays, and positive numbers are phase advances. (Light at CT12-18, wild-type: −3.18±0.41 h, n=6. *Cipc*$^{-/-}$: −3.71±0.46 h, n=10. Light at CT18-24, wild-type: 1.51±0.53 h, n=6. *Cipc*$^{-/-}$: 1.36±0.63 h, n=12).

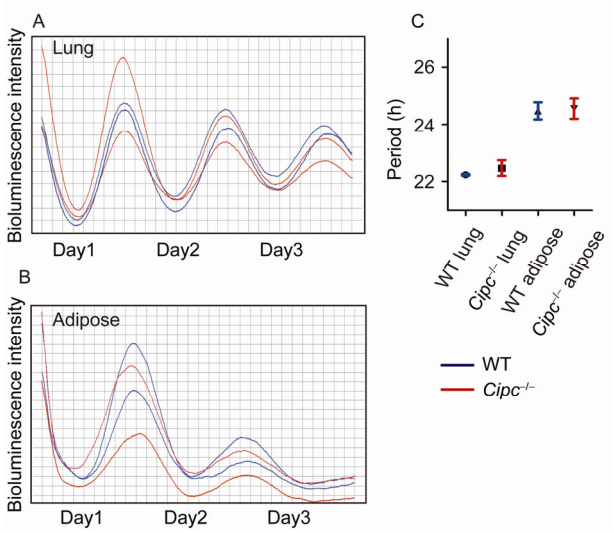

Figure 5 Oscillations of PER2:LUC in peripheral tissues from wild-type and *Cipc*$^{-/-}$ mice. Representative records of PER2:LUC bioluminescence rhythms in lung explants (A) and adipose explants (B). C, Quantification of the periods of lung and adipose explants for wild-type and *Cipc*$^{-/-}$ mice (Lung: WT: 22.23±0.03 h, n=3, *Cipc*$^{-/-}$: 22.47±0.27 h, n=3; P=0.214. Adipose: WT: 24.48±0.30 h, n=6, *Cipc*$^{-/-}$: 24.56±0.36 h, n=4; P=0.702).

3 Discussion

It has been shown that both deletion of endogenous *Cipc* and overexpression of *Cipc* in cultured cells led to shorter circadian periods. The loss-of-function and gain-of-function assays suggest that CIPC participates in circadian clock system. However, these assays were performed *in vitro* with some limitations to understand the functions of CIPC in an intact organism. As we have known, the best way to understand the functions of a gene is to edit this gene in animal level. Here, we generated *Cipc* knockout mice in which 352 aa of CIPC protein has been deleted, including CLOCK interacting domain. In the current study, we confirmed that CIPC does not function in the period determination in the locomotor rhythms. Putative CIPC paralogues were not found in our and other group studies [9], suggesting that lack of circadian phenotypes in *Cipc*$^{-/-}$ mice may not be due to functional redundancy of duplicated genes, but we could not exclude the possibility of complementation of other genes in *Cipc*$^{-/-}$ mice. The similar periods from peripheral tissues between wild type and *Cipc*$^{-/-}$ mice suggest that CIPC is not essential for timekeeping and period determination of circadian clock.

Previous studies suggest that loss of CIPC abolishes the inhibition on CLOCK-BMAL1 mediated E-box transactivation [9]. By contrast, the levels of most of E-box driven gene expressions are not elevated in *Cipc*$^{-/-}$ mice. Interestingly, though, we found that CIPC dampens the expression of *Per1* while other core clock genes were not significantly altered. These inconsistencies may result from tissue specific function or the difference between *in vitro* and *in vivo*. Of note, previous study showed the loss-of-function of CIPC in NIH3T3 cells [9], thus further studies should include monitoring the rhythms in various cell levels and assessing the complexity of physiological outputs of circadian clock.

This work was supported by the National Natural Science Foundation of China (31171343 and 31230049 to Xu Ying) and Ministry of Science and Technology (2006BAI23B00 to Xu Ying and Gao Xiang).

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